Application
for
United States Letters Patent

To all whom it may concern:

Be it known that Paul B. Fisher and Magdalena Leszczyniecka

have invented certain new and useful improvements in
GENES DISPLAYING ENHANCED EXPRESSION DURING CELLULAR SENESCENCE AND TERMINAL CELL
DIFFERENTIATION AND USES THEREOF

of which the following is a full, clear and exact description.

GENES DISPLAYING ENHANCED EXPRESSION DURING CELLULAR SENESCENCE AND TERMINAL CELL

5 ## DIFFERENTIATION AND USES THEREOF

This application claims priority and is a continuation-in-part application of U.S. Serial No. 09/243,277, filed February 2, 1999, the contents of which is hereby

10 incorporated by reference.

Throughout this application, various publications are referred to by arabic numeral within parentheses. Full citations for these publications are presented immediately

15 before the claims. Disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

20 ## BACKGROUND OF THE INVENTION

Normal cells cultured *in vitro* lose their proliferative potential after a finite number of doublings in a process described as cellular senescence (Hayflick and Moorehead, 1976). This phenomenon is not only well-established in human

25 diploid fibroblasts based on the studies of Hayflick and Moorehead (1976), but it has also been validated by investigations using many additional cell types (Goldstein *et al.*, 1990; Murano *et al.*, 1991). These investigations document an inverse correlation between replicative

30 senescence and donor age and a direct relationship between replicative senescence and donor species lifespan (Hayflick and Moorehead, 1976; Goldstein *et al.*, 1990; Murano *et al.*, 1991). In agreement with this association, cells from patients with premature aging syndromes, such as Werner's

35 syndrome and Progeria, achieve a quiescent state much more rapidly than normal human fibroblasts. In this context, if similar senescence related changes occur in normal fibroblasts, albeit with delayed kinetics, these cell systems represent excellent models for studying senescence *in vitro*

40 and identifying genes relevant to the aging process.

Senescence is characterized by changes in cell morphology, lack of responsiveness to mitogenic stimulation and irreversible growth arrest. However, cells can withdraw from the cell cycle and become non-dividing not only during

5 senescence but also during the processes of DNA damage, apoptosis or terminal differentiation. While senescence is a time-dependent process (Campisi et al., 1995), terminal differentiation can be induced in a variety of cell types by appropriate treatment (Roberts et al., 1999). For example,

10 terminal differentiation can be induced by cAMP treatment in melanocytes (Medrano et al., 1994). Gene expression analysis in terminally differentiated versus senescent melanocytes indicates both similarities and differences (Medrano et al., 1994). Although both pathways result in an elevation in p21

15 and an inability to phosphorylate ERK2, only the differentiated cells display elevated levels of p27 and the melanocyte-specific transcription factor (MITF) (Medrano et al., 1994; Smith and Pereira-Smith, 1996).

20 Human melanoma represents an excellent model for studying irreversible growth arrest and terminal differentiation, since these physiological changes can be chemically induced by IFN-β plus mezerein (MEZ) (Fisher et al., 1985; Jiang et al., 1994a). The induction of terminal differentiation in

25 HO-1 human melanoma cells correlates with up-regulation of c-jun, jun-B, α_5 Integrin, β_1 Integrin, fibronectin, HLA Class I, ISG-54, ISG-15 and gro/MSGA as well as down-regulation of c-myc (Jiang et al., 1993a). To define the repertoire of genes differentially expressed during induction of

30 irreversible growth arrest and terminal differentiation in human melanoma cells we have used a rapid and efficient differentiation induction subtraction hybridization (DISH) approach (Jiang and Fisher, 1993). Using this approach alone and in combination with high throughput screening strategies,

35 microchip DNA arrays, a large number of novel genes of potential relevance to growth control and terminal differentiation have been identified and cloned (Jiang et

al., 1995a, 1995b; Lin et al., 1996, 1998; Huang et al., 1999).

On the basis of the considerations described above, it is
5 probable that specific differentially expressed genes may be present within a terminally differentiated cDNA library that also display modified expression during cellular senescence. To begin to identify these overlapping genes, a temporally spaced subtracted·differentiation inducer treated HO-1 human
10 melanoma library was screened with RNA isolated from senescent human fibroblasts. Such a screening protocol yielded twenty-eight known and ten novel cDNAs. Subsequent Northern and reverse Northern blotting analyses revealed differential expression of specific cDNAs. Expression of one
15 of these cDNAs, Old-35 was restricted to terminal differentiation and senescence. In this context, this gene may contribute to pathways leading to growth arrest, a defining component of senescence and terminal differentiation.
20
Interferons (IFNs) comprise a family of related cytokines with diverse including antiviral, antiproliferative, antitumor and immunomodulatory activities (Stark et al., 1998; *Roberts et·al., 1999). IFN studies have focused on two
25 main areas; one involving the clinical use of IFN for therapeutic purposes (Gutterman, 1994), the other employing the IFN system as a paradigm to study the mammalian JAK/STAT signaling cascade (Darnell et al., 1994) that leads to IFN-stimulated gene (ISG) activation. To date, the most
30 extensively studied ISGs include RNA-activated protein kinase (PKR), the 2'-5' oligoadenylate synthetase and the MX proteins (Stark et al., 1998, *Der et al., 1998).

Post-transcriptional regulation of mRNA levels is a pivotal
35 control point in gene expression. Early response genes, such as cytokines, lymphokines and proto-oncogenes are regulated by a cis-acting adenylate-uridylate-rich element (ARE) found in the 3' untranslated region (UTR) of the mRNA (Caput et

al., 1986; Shaw and Kamen, 1988; Chen and Shyu, 1995; Myer
et al., 1997). Currently, three classes of destabilizing
elements have been identified: AUUUA-lacking elements and
AUUUA-containing elements grouped into those with scattered
5 AUUUA motifs (such as proto-oncogenes) and those with
overlapping AUUUA motifs (such as growth factors) (Chen et
al., 1995; Myer et al., 1997). A transfer of 3'UTR containing
ARE to 3'UTR of a stable message, such as β-globin, targets
this very stable mRNA for rapid degradation (Shaw and Kamen,
10 1988). In contrast, the removal of an ARE stabilizes an
otherwise labile message (*Miller et al., 1984; *Lee et al.,
1988).

The present studies describe the cloning and initial
15 characterization of a novel gene, Old-35, from a terminally
differentiated human melanoma cDNA library. mRNA stability
studies document that Old-35 mRNA, which contains ARE
elements, may be stabilized in HO-1 cells by treatment with
IFN-β and IFN-b + MEZ. Based on the growth suppressive effect
20 of IFN-β on HO-1 cells, as well as the increased stability
of Old-35 during confluence and senescence, it is possible
that this gene plays a prominent role in growth suppression
induced by this cytokine. Further experimentation is required
to define the precise role of Old-35 in IFN signaling,
25 terminal differentiation and cellular senescence. Full-length
cloning and subsequent protein analyses should provide
insights into the function of this potentially important gene
in the processes of aging and differentiation.

30 Since the processes of terminal differentiation and
senescence exhibit strikingly similar characteristics, it is
possible that related and overlapping genes and gene
expression changes associate with and mediate both of these
phenomena. Old-35 was isolated by screening a subtracted
35 human melanoma cDNA library enriched for genes related to
growth arrest and terminal differentiation with RNA from
senescent human fibroblasts. This cDNA encodes an IFN-β
inducible gene expressed during different types of growth

arrest including confluence, senescence and terminal differentiation. Old-35 RNA exhibits increased stability in IFN-β and INF-β + MEZ treated HO-1 human melanoma cells. Steady-state mRNA for Old-35 is also highly expressed in

5 heart and brain, human tissues without active regenerative properties. Judging from the pattern of Old-35 expression, it is possible that this gene may play a prominent role during growth arrest and in this context contributes to the important processes of senescence and terminal

10 differentiation.

SUMMARY OF THE INVENTION

This invention provides isolated nucleic acid molecule encoding an old 35 protein, 64 protein, 137 protein, 139 protein, 142 protein and a 175 protein. The isolated nucleic

5 acid may be a DNA, genomic DNA, cDNA, synthetic DNA or RNA. The isolated nucleic acid has a sequence substantially the same as SEQ ID. Nos. 39, 19, 31, 32, 34 and 38 which are respectively Old 35, old 64, old 137, old 139, old 142 and old 175.

10

This invention also provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding an old 35 protein, 64 protein, 137 protein,

15 139 protein, 142 protein and a 175 protein. The nucleic acid probe may be DNA, genomic DNA, cDNA, synthetic DNA or RNA.

This invention further provides a host vector system for the production of a protein having the biological activity of old

20 35, 64, 137, 139, 142 and 175. The isolated old 35, 64, 137, 139, 142 and 175 nucleic acid molecule is linked to a promoter of RNA transcription and then to a plasmid. The suitable host is a bacterial cell, insect cell, or animal cell, depending on the type of promoter and plasmid used.

25 This invention also provides a method of producing a protein having the biological activity of old 35, 64, 137, 139, 142 and 175, which comprises growing the selected host vector system under suitable conditions permitting production of the protein and recovering the protein so produced.

30

This invention further provides purified protein of old 35, 64, 137, 139, 142 and 175. Such purified old 35, 64, 137, 139, 142 and 175 will be useful for inhibiting growth of cancer cells. This invention provides a method of contacting

35 the cancer cells with an amount of old 35, 64, 137, 139, 142 and 175 at a concentration effective to inhibit growth of cancer cells. This invention further provides a method of determining whether a cell is senescent by (a)isolating the

nucleic acids in the cell   (b) hybridizing the isolated nucleic acids with the nucleic acid of old 35 or 64 under conditions permitting hybrids formation and (c) detecting the expression of old 35 or old 64 in the cell.   This invention

5 further provides a method of determining whether a cell has growth arrest by (a) isolating the nucleic acids in the cell; (b) hybridizing the isolated nucleic acids with the nucleic acid of old 35 or 64 under conditions permitting hybrids formation; and (c) detecting the expression of old 35 or old

10 64 in the cell. This invention further provides a method of determining whether a cell has terminal differentiation by (a) isolating the nucleic acids in the cell; (b) hybridizing the isolated nucleic acids with the nucleic acid of old 35 or 64 under conditions permitting hybrids formation; and (c)

15 detecting the expression of old 35 or old 64 in the cell. Further, this invention provides that the detector used is a DNA, RNA or protein. This invention also provides a method of regenerating tissue with an inhibitor of old 35 protein at a concentration effective to regenerate said tissues.

20 This invention provides a method of anti-aging in a cell comprising contacting the cell with an agent for inhibiting expression of old 35 at a concentration effective to reverse growth arrest in the cell. Finally, this invention provides a pharmaceutical composition for stimulating cell growth

25 comprising a pharmaceutically acceptable carrier and purified old 35 or old 64 .at a concentration effective to stimulate cell growth.

BRIEF DESCRIPTION OF FIGURES

Figure 1 Expression of Old-35 in HO-1 human melanoma cells treated with IFN-β or FN-β + MEZ, young human fibroblasts and two different types of senescent Progeria human fibroblasts. Northern blot contains 10μg of total RNA from control untreated HO-1 (lane 1), IFN-β treated (2,000 U/ml) HO-1 (lane 2), IFN-β + MEZ treated (2,00U/ml + 10ng/ml) HO-1 (lane 3), young fibroblasts (GM01379) (lane 4), and two senescent Progeria cell lines (AG01976) (lane 5) (AG0989B) (lane 6). Blots were exposed for autoradiography for 1, 4 or 24 hr. EtBr staining for quantification of gel loading and determining RNA quality.

Figure 2 Effect of IFN-α, IFN-β, IFN-γ, TNF-α and IFN-β + MEZ on Old-35 expression in HO-1 cells. All Northern blots contain 10 mg of total RNA. (A) Time course induction of Old-35 by IFN-β in HO-1 cells. Cells were seeded at ~60% confluence and treated with IFN-β (2,000 units/ml) and RNA was isolated at the indicated time. U = RNA from control, untreated cells. (B) Dose response expression of Old-35 in HO-1 cells treated with IFN-β (2,000 units/ml). RNAs were isolated after 24 hr treatment. (C) Effect of IFN-α (Iα), IFN-β (Iβ), IFN-γ (Iγ) and TNF-α (Tα) on Old-35 expression in HO-1 cells. RNAs were isolated after 15 hr treatment with 1,000 units/ml of the different agents. U = RNA from control, untreated cells. (D) Time course induction of Old-35 by IFN-β + MEZ in HO-1 cells. RNAs were isolated from cells treated with 2,000 units/ml of IFN-β + 10 ng/ml of MEZ.

Figure 3 Expression of Old-35 in various human tissues and during mouse development. (A) Northern blot

contains 2µg of poly A⁺ RNA per lane from eight different human tissues. Lanes 1-8 contain, in order, RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Clontech). (B) Northern blot contains 10µg of total RNA from mouse embryos. The number of days signifies days post-gestation.

Figure 4 Sequence comparison between human and the mouse homologue of Old-35. Upper panel sequence of human Old-35 (h-Old-35); Middle panel: sequence of mouse Old-35 (m-Old-35); and Lower panel: shared consensus sequences between human and mouse Old-35.

Figure 5 Expression of Old-35 in IDH4 cells grown in the presence or absence of Dex. Northern blot contains 10µg of total RNA per lane from IDH-4 cells. + Dex = cells grown continuously in the presence of 10^{-6} M Dex; - Dex = cells grown for the indicated days in the absence of Dex. For the latter experiment, cells were grown in the presence of Dex and then shifted to charcoal stripped media and grown for 3, 5, 7 and 14 days without Dex.

Figure 6 Expression of Old-35 and p21 during cell cycle progression in human skin fibroblasts. Northern blot contains 10µg of total RNA per lane from normal human fibroblasts. Confluent normal fibroblasts (C) were trypsinized and reseeded (1:2). Total RNA was collected at 5, 15 and 20 hr after reseeding. At 20hr following subculture, the cells were 90% confluent.

Figure 7 AU rich sequences found in the 3' untranslated region (UTR) of several lymphokine and

protooncogene mRNAs. Abbreviations: Abbreviations: Hu-human, GM-CSF = granulocyte-monocyte colony stimulating factor; IFN-α = interferon α; IL 2 = Interleukin 2; TNF = tumor necrosis factor; c-fos = fos proto-oncogene. The underlined/overlined AUUUA motif if the largest sequence common to all mRNAs shown. References: HuGM-CSF (Wong et al., 1985), HuIFN-α (Goeddel et al., 1983), Hu IL 2 (Kashima et al., 1985), HuTNF (Nedwin et al., 1985), Hu c-fos (van Straaten et al., 1983).

Figure 8   Effect of cycloheximide treatment on Old-35 expression in HO-1 cells and the half-life of Old-35 mRNA in IFN-β + MEZ treated HO-1 cells. Each lane in the Northern blots contains 10μg of total RNA. (A) HO-1 cells were pre-treated with cyclohexamide 50mg/ml for 30 min and then treated with IFN-β for 2, 3 or 4 hr (**lanes 2, 3, and 4, respectively**). HO-1 cells were pre-treated with IFN-β for 5 hr (**lane 5**) and then treated with cycloheximide for 15 hr (**lane 6**). U = RNA from control untreated HO-1 cells. (B) Half-life of Old-35 mRNA in IFN-β + MEZ (IM) (2,000 units/ml + 10 ng/ml) treated HO-1 cells. Cells were incubated with IM for 15 hr and then exposed to ActD (50 mg/ml) for 2, 6, 8, 10 and 12 hr. U = RNA from control untreated HO-1 cells. AD = RNA from control HO-1 cells treated with ActD (5 μg/ml).

Figure 9   DNA sequence and predicted encoded protein of Old-35. (A) cDNA sequence of Old-35. Alternate polyadenylation site is underlined. This site is present in 10% of all cDNAs (*Manley et al., 1988). (B) Predicted protein encoded by the Old-35 cDNA.

Figure 10   Sequence similarity between the bacterial protein PNPase and the predicted protein sequence of Old-35. Upper Panel: *Bacillus subtilis* PNPase sequence. Middle Panel: predicted human Old-35 protein sequence. Lower Panel: regions of consensus amino acids between the bacterial PNPase protein sequence and the predicted Old-35 protein sequence. Black boxed areas indicates amino acid identity and gray boxed areas indicate amino acid similarities between the bacterial PNPase and the predicted Old-35 encoded protein.

Figure 11   Northern Blot of HO-1, confluent HO-1, IFN-β treated, IFN-β+MEZ treated HO-1 treated with Actinomycin D (50mg/ml). Total RNAs were collected 2,4,6,8,10,12 after the AD treatment. Old-35 cDNA was used as a probe. Ethydium Bromide was shown for loading control

Figure 12   Northern Blot of IDH4 and AR5 cells. IDH4 cells contain dexamethasone (DEX) inducible mouse mammary tumor virus-driven simian virus 40 T-antigen. Total RNA was extracted from cells treated with DEX (indicated as +), and from cells growing without DEX for 3,5,7, and 14 days). AR5 cells contain temperature sensitive simian virus 40 T-antigen. Total RNA was collected from cells at 35C and 1,3,7,14 days after shift to 39C. Old-35 and p21 were used as a probe.

Figure 13   Structure of Old-35 gene. RnasePH, KH, S1 signify domains found in Old-35 cDNA. Top picture shows two different versions of Old-35 which vary in the 3'UTR length
The bottom picture shows cloning of the Old-35 cDNA using C-ORF technique.

Figure 14

Localization of GFP-Old-35, and GFP alone in HeLa cells.

Figure 15

5 *In situ* hybridization to mouse embryo (9.5 days) using murine Old-35. The arrows indicate the expression in the spinal column.

Figure 16

10 Northern blot of HO-1 cells treated with different subtypes of IFN-α using Old-35 as a probe. IFN-β was used as a control.

DETAILED DESCRIPTION OF THE INVENTION

In order to facilitate an understanding of the Experimental Details section which follows, certain frequently occurring methods and/or terms are described in Sambrook, et al.
5 (1989).

Throughout this application, the following standard abbreviations are used throughout the specification to indicate specific nucleotides:

10   C=cytosine   A=adenosine
         T=thymidine   G=guanosine

This invention provides an isolated nucleic acid molecule encoding an OLD-35 or OLD-64 protein. In an embodiment, the
15 above nucleic acid molecule comprises a nucleic acid having a sequence substantially the same as set forth in SEQ. ID. No.39 or 19.

This invention also provides isolated nucleic acid molecules
20 encoding an OLD-137, OLD-139, OLD-142, or OLD-175 protein. In an embodiment, the nucleic acid comprises a nucleic acid having a sequence substantially the same as set forth in SEQ. ID. Nos.31, 32, 34 or 38. The above-described nucleic acid may be DNA, genomic DNA, cDNA, synthetic DNA, or RNA.
25
This invention also encompasses nucleic acid which encode amino acid sequences which differ from those of OLD-35, OLD-64, OLD-137, OLD-139, OLD-142 or OLD-175, but which should not produce phenotypic changes. Alternatively, this
30 invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the subject invention. Hybridization methods are well known to those of skill in the art.

The DNA molecules of the subject invention also include DNA
35 molecules coding for protein analogs, fragments or derivatives of antigenic proteins which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs

containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a
5 terminal or medial portion of the proteins) and which share some or all properties of naturally-occurring forms. These sequences include: the incorporation of codons "preferred" for expression by selected non-mammalian host; the provision of sites for cleavage by restriction endonuclease enzymes;
10 and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The nucleic acid molecule described and claimed herein are
15 useful for the information which they provide concerning the amino acid sequence of the protein and as products for the large scale synthesis of the protein by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed
20 and transfected procaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the protein and related products.

The invention also provides fragments or portion of the Old
25 gene or protein wherein the biological activity of said gene product is maintained. Such fragment or portion may join to other amino acid sequence to create a multi-functional molecule. It is within the ordinary skill to determine such biologically active fragment or portion. A trimming
30 experiment may be performed to define said fragment of portion.

Old-35, Old-64, Old-137, Old-139, Old-142 or Old-175 may be isolated in a variety of vertebrates. In an embodiment, a
35 human Old-35, Old-64, Old-137, Old-139, Old-142 and Old-175 are isolated.

The isolated nucleic molecule of Old-35, Old-64, Old-137,

Old-139, Old-142 and Old-175 are represented respectively by SEQ. ID. Nos. 39, 19, 31, 32, 34 and 38.

This invention provides a nucleic acid molecule of at least
5 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a Old-35, Old-64, Old-137, Old-139, Old-142 or Old-175. In an embodiment, the nucleic acid is DNA, genomic DNA, cDNA, synthetic DNA or RNA.
10

As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between
15 complementary base pairs. The nucleic acid molecule will be specific to said Old genes i.e. under appropriate conditions, the molecule will only hybridize with said old gene and no other genes. Said molecule may contain an unique sequence of said Old gene.
20

Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to
25 facilitate detection of the probe.

Probe molecules may be produced by insertion of a nucleic acid molecule which encodes OLD-35, OLD-64, OLD-137, OLD-139, OLD-142 or OLD-175 protein or a fragment thereof into
30 suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from
35 DNA synthesizers.

The probes are useful for 'in situ' hybridization to locate tissues which express this gene, or for other hybridization

assays for the presence of this gene or its mRNA in various biological tissues.

The invention also provides an antisense nucleic acid
5 molecule comprising a sequence complementary to the nucleic acid which encodes OLD-35, OLD-64, OLD-137, OLD-139, OLD-142 or OLD-175 protein or a fragment thereof. In an embodiment, the antisense nucleic acid molecule is capable of inhibiting the expression of the hybridized gene.
10

This invention also provides the above-described isolated nucleic acid molecule operatively linked to a promoter of RNA transcription. This invention further provides a vector which comprises the above-described isolated nucleic acid
15 molecule.

Vectors which comprise the isolated nucleic acid molecule described hereinabove also are provided. Suitable vectors comprise, but are not limited to, a plasmid or a virus.
20 These vectors may be transformed into a suitable host cell to form a host cell vector system for the production of a protein having the biological activity of OLD-35, OLD-64, OLD-137, OLD-139, OLD-142 or OLD-175 protein or a fragment thereof.
25

This invention further provides an isolated DNA, genomic DNA , cDNA, synthetic DNA or RNA molecule described hereinabove wherein the host cell is selected from the group consisting of bacterial cells (such as E.coli), yeast cells, fungal
30 cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

This invention provides a purified, OLD-35 protein, a
35 purified, OLD-64 protein, a purified, OLD-137 protein, a purified, OLD-139 protein, a purified, OLD-142 protein, and a purified, OLD-175 protein.

This invention also provides a protein encoded by the above-described isolated nucleic acid molecule.

This invention also provides an antibody or antigen-binding
5 fragment thereof that specifically binds to OLD-35, OLD-64, OLD-137, OLD-139, OLD-142 or OLD-175 protein. In an embodiment, the antibody is a monoclonal antibody.

Polyclonal antibodies against these proteins may be produced
10 by immunizing animals using selected peptides determined from the decoded amino acid sequences. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the
15 desired antibody. Alternatively, monoclonal antibodies may be produced by _in vitro_ techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of the OLD proteins in living animals, in humans, or in biological tissues or fluids isolated from
20 animals or humans.

This invention provides a method of inhibiting growth of cancer cells comprising contacting the cancer cells with an amount of purified OLD-35, OLD-64 protein or a portion
25 thereof effective to inhibit growth of cancer cells.

This invention also provides a method for reversing the cancerous phenotype of a cancer cell which comprises introducing a nucleic acid comprising an Old-35 or Old-64
30 gene or a portion thereof into the cell under conditions permitting the expression of the gene so as to thereby reverse the cancerous phenotype of the cell.

This invention provides a method for reversing the cancerous
35 phenotype of a cancer cell in a subject which comprises introducing a nucleic acid molecule comprising an Old-35 or Old-64 gene or a portion thereof into the subject's cancerous cell under conditions permitting expression of the gene in

the subject's cell so as to thereby reverse the cancerous phenotype of the cell.

In an embodiment of the method, the nucleic acid molecule
5 comprises a vector. In a further embodiment, the Old-35 or Old-64 gene is linked to a regulatory element such that its expression is under the control of the regulatory element. In a still further embodiment, the regulatory element is a tissue specific regulatory element. In a still further
10 embodiment, the regulatory element is inducible or constitutive. Inducible regulatory element like an inducible promoter is known in the art. Regulatory element such as promoter which can direct constitutive expression is also known in the art.
15

In a separate embodiment, the regulatory element is a tissue specific regulatory element. The expression of the inserted gene will then be tissue-specific.

20 Methods to introduce a nucleic acid molecule into cells have been well known in the art. Naked nucleic acid molecule may be introduced into the cell by direct transformation. Alternatively, the nucleic acid molecule may be embedded in liposomes. Accordingly, this invention provides the above
25 methods wherein the nucleic acid is introduced into the cells by naked DNA technology, adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retroviral vectors, vaccinia virus vector, liposomes, antibody-coated liposomes, mechanical or
30 electrical means. The above recited methods are merely served as examples for feasible means of introduction of the nucleic acid into cells. Other methods known may be also be used in this invention.

35 This invention provides a method for reversing the cancerous phenotype of a cancer cell which comprises introducing OLD-35 or OLD-64 protein or a portion thereof into the cancerous cell so as to thereby reverse the cancerous phenotype of the

cell.

This invention provides a method for reversing the cancerous phenotype of a cancer cell in a subject which comprises introducing OLD-35 or OLD-64 protein into the subject's cancerous cell so as to thereby reverse the cancerous phenotype of the cell. In an embodiment, the cancer cell is a breast, cervical, colon, pancreatic, thyroid, skin, brain, prostate, nasopharyngeal, lung, glioblastoma multiforme, lymphoma, leukemia, connective tissue, nervous system cell or basal cell.

This invention further provides a pharmaceutical composition which comprises an amount of a nucleic acid molecule comprising Old-35, Old-64 gene or portion thereof effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier. In an embodiment, the nucleic acid molecule comprises a vector. In a further embodiment, the vector is an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retrovirus vector or vaccinia virus vector.

This invention also provides a pharmaceutical composition comprising an amount of OLD-35 or OLD-64 protein effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier. In an embodiment, the cancer cell is a breast, cervical, colon, pancreatic, thyroid, skin, brain, prostate, nasopharyngeal, lung, glioblastoma multiforme, lymphoma, leukemia, connective tissue, nervous system or basal cell.

In an embodiment of the above methods, the nucleic acid comprises a vector. The vector includes, but is not limited to, an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retrovirus vector and vaccinia virus vector.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers. The pharmaceutical composition may be constituted into any form suitable for the mode of administration

5 selected. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions,

10 emulsions, and suspensions.

In the practice of the method administration may comprise daily, weekly, monthly, hourly or by peak and trough, the precise frequency being subject to various variables such

15 as age and condition of the subject, amount to be administered, half-life of the agent in the subject, area of the subject to which administration is desired and the like.

In connection with the method of this invention, a

20 therapeutically effective amount may include dosages which take into account the size and weight of the subject, the age of the subject, the severity of the symptom, the efficacy of the agent and the method of delivery of the agent. One of ordinary skill in the art would be readily able to determine

25 the exact dosages and exact times of administration based upon such factors.

This invention provides a method of determining whether a cell is senescent comprising measurement of the expression

30 of Old-35 gene, wherein the expression of the Old-35 gene indicates that the cell is senescent. In an embodiment, the expression of the Old-35 gene is measured by the expression of Old-35 specific RNA. In another embodiment, the expression of the Old-35 gene is measured by the expression

35 of the OLD-35 protein.

This invention also provides a method of determining whether a cell is terminally differentiated comprising measurement

of the expression of Old-35 gene, wherein the expression of the Old-35 gene indicates that the cell is terminally differentiated. In an embodiment, the expression of Old- 35 gene is measured by the expression of Old-35 specific RNA.

5 In another embodiment, the expression of the Old-35 is measured by the expression of OLD-35 protein.

This invention provides a method of determining whether a cell has growth arrest comprising measurement of the

10 expression of Old-35 gene, wherein the expression of Old-35 gene indicates that the cell has growth arrest. In an embodiment, the expression of the Old-35 gene is measured by the expression of old 35 specific RNA. In another embodiment, the expression of the Old-35 gene is measured by

15 the expression of OLD-35 protein.

This invention provides a method of inhibiting growth of cancer cells comprising contacting the cancer cells with an amount of purified OLD-64 protein effective to inhibit growth

20 of cancer cells.

This invention also provides a method of inhibiting growth of cancer cells comprising contacting the cancer cells with an amount of purified OLD-64 protein effective to inhibit

25 growth of cancer cells.

This invention provides a method of determining whether a cell is senescent comprising measurement of the expression of Old-64 gene, wherein the expression of the Old-64 gene

30 indicates that the cell is senescent. In an embodiment, the expression of old-64 gene is measured by the expression of Old-64 specific RNA. In another embodiment, the expression of Old-64 gene is measured by the expression of the OLD-64 protein.

35

The expression of specific OLD RNA may be measured by the below method: (a) isolating the nucleic acids from a sample; (b) hybridizing the isolated nucleic acids with the

appropriate Old gene under conditions permitting hybrids formation; and (c) detecting the hybrid formed.

The invention provides a pharmaceutical composition for
5 inhibiting cell growth comprising a pharmaceutically acceptable carrier and purified old 35 or old 64 at a concentration effective to inhibit cell growth.

This invention provides a method of determining whether a
10 cell is terminally differentiated comprising measurement of the expression of Old-64 gene, wherein the expression of the Old-64 gene indicates that the cell is terminally differentiated. In an embodiment, the expression of Old-64 gene is measured by the expression of Old-64 specific RNA.
15 In another embodiment,the expression of Old- 64 gene is measured by the expression of the OLD-64 protein.

This invention provides a method of determining whether a cell is growth arrested comprising measurement of the
20 expression of Old-64 gene, wherein the expression of Old-64 gene indicates that the cell is growth arrested. In an embodiment, the expression of Old- 64 gene is measured by the expression of Old-64 specific RNA. In another embodiment, the expression of Old- 64 gene is measured by the expression
25 of the OLD-64 protein.

This invention provides a method of regenerating tissues comprising contacting the tissue with an inhibitor of OLD-35 or OLD-64 protein at a concentration effective to regenerate
30 said tissues.

Methods to determine such a concentration are well-known in the art. The effective concentration of said inhibitor of OLD-35 or OLD-64 protein may be determined by using different
35 concentrations of said inhibitor and examine the effect produced.

This invention provides a method of anti-aging in a cell

comprising contacting the cell with an agent for inhibiting expression of Old-35 or Old-64 gene at a concentration effective to reverse the aging process in the cell.

5 This invention provides a pharmaceutical composition for stimulating or resuming cell growth comprising a pharmaceutically acceptable carrier and purified Old-35 or Old-64 suppressant at a concentration effective to stimulate or resuming cell growth. A purified suppressant is compound 10 capable of suppressing the activity of OLD-35 or OLD-64. For example, the suppressant can act on the gene level such that no Old-35 or Old-64 gene will be switched on. Alternatively, the suppressant may be a samll molecule capable of binding to the active sites on the OLD-35 or -64 protein such that 15 the protein will not be functional or the activity of the protein will decrease. A specific antibody or its binding fragment, which is capable of binding to the OLD-35 or -64, may be a suppressant.

20

This invention provides a method for screening the presence of interferon alpha or beta of a sample comprising steps of:(a) contacting the sample with cells under conditions permitting expression of Old-35 or Old-64 gene in the 25 presence of interferon alpha or beta; and (b) determining the expression of Old-35 or Old-64 gene, an increase of expression indicates the presence of interferon alpha or beta.

30 This invention provides a method for detection of the secretion of interferon alpha or beta comprising steps of: (a) obtaining an appropriate sample from the subject; and (b) detecting expression of Old-35 or Old-64 gene, the expression of Old-35 or Old-64 gene indicating the secretion of 35 interferon in a subject.

This invention provides a method for monitoring chemotherapy of a subject comprising steps of: (a) obtaining an

appropriate sample from the subject; and (b) detecting expression of Old-35 or Old-64 gene, the expression of Old-35 or Old-64 gene indicating that the chemotherapy is effective.

5 This invention provides a method for diagnosis of the proliferating stage of a tumor from a subject comprising steps of:(a) obtaining an appropriate sample from the subject; and (b) detecting expression of Old-35 or Old-64 gene, the expression of Old-35 or Old-64 gene indicating that 10 the tumor is not at a proliferating stage.

This invention also provides a kit for diagnosis of the proliferating stage of a tumor, comprising a nucleic acid molecule capable of specifically hybridizing to the nucleic 15 acid molecule of Old-35 or Old-64.

This invention also provides a kit for diagnosis of the proliferating stage of a tumor, comprising antibody capable of specifically recognizing OLD-35 or OLD-64 protein.
20
This invention provides different kits containing appropriate reagents to perform the above-described methods.

This invention also provides a method for identifying an 25 agent that modulates the expression of Old-35 or Old-64 gene, comprising: (a)contacting a candidate agent with a cell transformed or transfected with a reporter gene under the control of a Old-35 or Old-64 promoter or a regulatory element thereof under conditions and for a time sufficient 30 to allow the candidate agent to directly or indirectly alter expression of the promoter or regulatory element thereof; and (b) determining the effect of the candidate agent on the level of reporter protein produced by the cell, thereby identifying an agent that modulates expression of Old-35 or 35 64 gene.

This invention provides a method of identifying compounds that induce proliferation or cancerous phenotype,

comprising: exposing cell comprising the promoter of Old-35 or Old-64 to the compound and identifying compounds that suppress the Old-35 or 64 promoter.

5 This invention provides a method of identifying compounds that induces senescence, or terminal differentiation, comprising: exposing the cell comprising the promoter of Old-35 or Old-64 ·to the compound and identifying compounds that activate the Old-35 or 64 promoter.

10

This invention provides a method of identifying genes which are common to the pathway of senescence and terminal differentiation comprising steps of: (a) obtaining a subtracted library which is enriched for genes expressed in

15 terminal differentiation; (b) screening the library with senescent probe to identify novel genes which are expressed during senescence and terminal differentiation; and (c) examining the biological activity of the identified gene to determined whether it is expressed during senescence and

20 terminal differentiation.

This invention provides a method of identifying genes which are common to the pathway of senescence and terminal differentiation comprising steps of: (a) obtaining a

25 subtracted library which is enriched for genes expressed in senescence; (b) screening the library with terminal differentiation probe to identify novel genes which are expressed during senescence and terminal differentiation; and (c) examining the biological activity of the identified gene

30 to determined whether it is expressed during senescence and terminal differentiation.

This invention also provides the gene identified by the above methods.

35

This invention provides a method of degrading specific RNAs in a cell comprising induction of the expression of Old-35. This invention further provides a method of degrading

specific RNAs in a cell comprising introducing a vector into the cell comprising the Old-35 gene.

In one embodiment of the invention, expression of Old-35 can
5 be used as diagnostic indicator of cellular senescence, terminal differentiation and/or growth suppression. Specifically, Old-35 can be used to determine if a cell has lost proliferative ability and thus has become senescent.

10 In addition, expression of Old-35 can be used as a marker to identify drugs or small molecules that will induce senescence, e.g., to inhibit cancer cell growth or abnormal proliferative states such as psoriasis, hemangioblastoma, etc..
15

Further, expression of Old-35 can be used to identify drugs or small molecules that will inhibit senescence, and thus stimulate tissue regrowth, repair and/or regeneration.

20 Still further, expression of Old-35 can be used as a marker to identify drugs or small molecules that will induce terminal cell differentiation, e.g., to inhibit cancer cell growth or abnormal proliferative states such as psoriasis, hemangioblastoma, etc..
25

Expression of Old-35 can also be used to identify drugs or small molecules that will inhibit terminal differentiation, and thus stimulate tissue regrowth, repair and/or regeneration.
30

Furthermore, expression of Old-35 can be used as marker for detecting cytokines, specifically type I interferons, in biological samples. Since type I interferons, including leukocyte and fibroblast interferons, which activate gene
35 expression through the well characterized Jak and Stat kinase pathways, this gene (Old-35) can be used to detect or monitor drugs and other small molecules that activate these important pathways.

The combination of Old-35 with other interacting proteins can be used to target the differentiation of specific target cells, and thus result in the reprogramming of pluripotent stem cells to terminally differentiated end cells.

5

Additionally, Old-35 can be used to selectively stabilize specific mRNAs possibly containing AU rich 3' UTRs (untranslated regions). This effect can result in the sustained expression of genes potentiating or inhibiting cell

10 growth. It could also result in the stabilizing of cytokine genes resulting in increased biological and immunological activity.

Old-35 can also be used as part of a methodology to

15 polymerize random NTPs into nucleic acids and/or to induce the degradation of specific mRNAs.

This invention will be better understood from the Experimental Details which follow. However, one skilled in

20 the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Library Screening

A subtracted cDNA library enriched in genes modified during
5 terminal differentiation in human melanoma cells (Jiang and
Fisher, 1993) was plated at 200 pfu/plate. Colonies were
transferred to Nylon filters, denatured for 2 min (1.5M NaCl,
0.5M NaOH), neutralized for 5 min (1.5M NaCl, 0.5M Tris-HCl,
pH 8.0), and washed for 30 sec (0.2M Tris-HCl, pH 7.5, 2 X
10 SSC). Filters were cross-linked (120,000 μJ of UV energy) for
30 sec in a Strata linker (Stratagene) and prehybridized at
65°C for 2 hr in ExpressHyb (ClonTech). The probe was
denatured at 95°C for 5 min, cooled at 0°C for 5 min and then
applied to the filters at 3 X 10^6 cpm/ml. The filters were
15 hybridized overnight at 65°C. The next day, the filters were
washed (2 X SSC, 0.1 % SDS) 3 X for 20 min and exposed for
autoradiography.

PREPARATION OF THE PROGERIA CDNA PROBE

20 Ten μg of total RNA derived from AG0989B cells (Progeria) (p
22) (Corriel Repository, Camden) was reverse transcribed
using SuperScript II (manufacturer's protocol, GibcoBRL)
except that 900 μCi of [α-^{32}P]-dCTP (3000Ci/mmole) (Amersham)
and 0.4mM of non-radioactive dCTP was used in place of 10mM
25 dCTP. The probe was purified using Quick Spin Columns
(Boehringer Mannheim).

PHAGE ISOLATION

The exposed film from autoradiography was aligned with the
30 phage containing plates and hybridizing clones were isolated
and re-suspended in SM buffer (1 ml).

PCR

PCR was performed for each phage stock using the
35 manufacturer's protocol (GibcoBRL) with 3 μl of SM stock.
Since T3 and T7 primers flank the insert, these primers were
used to selectively amplify the insert from the phage vector

(Stratagene). PCR conditions were 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and 72°C for 10 min to allow complete extension. The PCR products were resolved on 1% agarose gels to determine the size of the product. All clones were sequenced and the novel cDNAs were selected for Northern blotting analysis.

NORTHERN BLOTTING

Total RNA was extracted using the guanidinium isothiocyanate method followed by phenol/chloroform/isoamyl extraction and precipitation in isopropanol as described in Chomczynski and Sacchi (1987). The probes were labeled with $[\alpha-^{32}P]dCTP$ by random priming (Amersham). Ten μg of total RNA were electrophoresed in a 1% agarose/2.2M formaldehyde gel and transferred to Hybond-NX filters (Amersham). Hybridization was performed in ExpressHyb solution (Manufacturer's protocol, Clontech). Briefly, filters were prehybridized at 67°C for 0.5 hr, hybridized with a denatured probe for 1.5 hr, and washed (.2 X SSC, .1 % SDS) 1 X at 24°C for 5 min, and 2 X at 55°C for 20 min.

CELLS AND CULTURE CONDITIONS

HO-1 human melanoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum at 37°C in a 5% CO_2/95% air humidified incubator. Cell lines used for the senescence study were obtained from Corriel Repository (Camden, NJ). Fibroblast cell lines from patients with Progeria-Hutchinson-Gilford Syndrome (AG01972B, AG0989B, AG01178B) and normal fetal fibroblasts (GM01379A) were grown in DMEM supplemented with 15% fetal bovine serum (Gibco BRL) and 2 X essential and non-essential amino acids (Sigma). IDH4 cells (Wright et al., 1989) were grown in DMEM supplemented with 10% fetal bovine serum or 10% charcoal stripped fetal bovine serum. HO-1 cells were treated with IFN-β (2,000 U/ml) and MEZ (10 ng/ml) to induce terminal differentiation (Fisher et al., 1985). To inhibit RNA and protein synthesis, HO-1 cells were treated with actinomycin D (5 μg/ml) and

cycloheximide (50 μg/ml), respectively, as previously described (Jiang et al., 1993b).

STAINING FOR SENESCENCE-ASSOCIATED (SA) B-GAL ACTIVITY

5 Cells were washed 2 X with PBS, fixed in 3% formaldehyde, and stained as previously described (Dimri *et al.*, 1995). Briefly, following fixation, cells were incubated overnight at 37°C in a reaction buffer containing X-gal (1 mg/ml), 40mM citric acid/sodium phosphate (pH 6.0), potassium

10 ferrocyanide/ferricyanide (5mM), NaCl (150mM) and 2mM MgCl$_2$. IDH4 cells grown in the presence of dexamethasone (10^{-6} M) were used as a negative control.

EXPERIMENTAL RESULTS

15 Preliminary screening of cDNA libraries screening the temporally spaced subtracted differentiation inducer treated HO-1 cDNA (DISH) library enriched for genes regulated during terminal differentiation in melanoma cells, with the RNA from senescent fibroblasts, resulted in the identification of 10

20 novel and 28 known cDNAs, referred to as Old cDNAs (Table 1). Northern and reverse Northern blotting was used to determine the expression patterns of these Old cDNAs. The goal of our screening was to identify and clone differentially expressed genes common to senescence and terminal differentiation. To

25 achieve this aim, RNAs from HO-1 (untreated or treated with IFN-β, 2,000 U/ml or IFN-β (2,000 U/ml) +MEZ (10 ng/ml)), young fibroblast cultures (GM01379) and two senescent cell cultures (AG01976, AG0989B) were isolated and expression of specific Old genes was determined (Fig. 1). Since the

30 subtracted library that was screened should be enriched for HO-1 genes regulated by IFN-β and IFN-β + MEZ, it was anticipated that the level of expression of many of the Old cDNAs would be reduced or absent in actively proliferating, untreated HO-1 cells. However, since this library was

35 screened with an un-subtracted senescent probe (containing senescent specific, housekeeping and other genes) some of the cDNAs should also be expressed in non-senescent fibroblasts.

Four of the six novel cDNAs, Old-137, Old-139, Old-142 and Old-175, were expressed in both proliferating and senescent fibroblasts. Expression of two novel Old genes, Old-35 and Old-64, were restricted to the senescent fibroblasts and

5 IFN-b and IFN-β + MEZ treated H0-1 cells. Different exposure times revealed that the expression level of Old-35 is higher in senescent fibroblasts than in HO-1 cells treated with IFN-β or IFN-β + MEZ (Fig. 1). Response of Old-35 to Interferons Time-course and dose-response experiments were

10 performed in HO-1 cells to determine the temporal kinetics of Old-35 induction by IFN-β and the concentration of IFN-β capable of inducing Old-35 expression, respectively. Additionally, the effect of IFN-α, IFN-γ and TNF-α on Old-35 expression in HO-1 cells was examined. Old-35 was

15 up-regulated by IFN-β (2,000 units/ml) and IFN-β + MEZ (2,000 units/ml + 10 ng/ml) within 3 hr of treatment (Fig. 2A and D). Since IFN-β induces growth suppression in HO-1 cells at 2,000 units/ml, it was considered important to determine whether up-regulation of Old-35 could occur in the absence

20 of growth suppression. Old-35 expression was induced in HO-1 cells with as little as 1 U/ml of IFN-α, a dose of IFN that is not growth inhibitory, suggesting a direct effect of IFN on expression of this gene in the absence of growth suppression (Fig. 2B). Treatment of HO-1 cells with IFN-α

25 resulted in significant up-regulation of Old-35 in HO-1 cells, whereas this expression was marginally stimulated by IFN-γ and no detectable or consistent induction occurred with TNF-α (Fig. 2C). These experiments document differential regulation of Old-35 expression by different cytokines, with

30 type I Interferons (IFN-α/IFN-β) being the most active cytokines tested in inducing Old-35 expression in HO-1 cells. Expression of Old-35 in various human tissues and during mouse development to determine the tissue-specific expression pattern of Old-35 we examined the expression of this gene

35 using Human Multiple Tissue Northern (MTN) Blots (Clontech) (Fig. 3 A). Old-35 was expressed in all of the tissues tested, including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The highest levels of

Old-35 expression were detected in the heart and brain. Since the heart and brain contain a high proportion of non-regenerating, terminally differentiated cells, it is possible that Old-35 may be important in maintaining end
5 stage differentiation in these target organs. Since terminal differentiation of specific tissue cell types occurs during normal development of the embryo, the expression pattern of Old-35 was determined during mouse development. The highest level of Old-35 expression was apparent during the earliest
10 stage of development (8 days) and it steadily declined with time (10 to 16 days) (Fig. 3B). This dilution effect is frequently observed when mRNA expression is localized in a specific organ as the embryo develops, because the ratio of the region of expression to the whole body decreases over
15 time. Since the mouse developmental Northern Blot was probed with human cDNA and the resulting signal was very strong, the homology between human and mouse OLD-35 transcripts must be very high. The EST database search showed very close homology between the mouse and the human cDNA, ~ 90% (Fig. 4).
20 Expression of Old-35 during growth arrest and senescence in IDH4 cell IDH4 cells were produced by transfecting IMR-90, normal human fibroblasts, with a dexamethasone (DEX) inducible mouse mammary tumor virus-driven simian virus 40 T-antigen (Wright et al., 1989). In this model system,
25 prolonged proliferation and the absence of markers of senescence are dependent upon the continued presence of DEX and thus the SV40 T-antigen. (Wright et al., 1989). In DEX-free medium, DNA synthesis declines by ~80% within the first 3 days and reaches a minimum level at day 7. This
30 decline corresponds with a decrease in telomerase activity and T-antigen expression (Holt et al., 1996). Since T-antigen has a long half-life (~3 days) and remains in the cells for about 5-7 days after the removal of DEX, it is possible that the up-regulation of Old-35 by day 7
35 corresponds with the depletion of T-antigen in these cells (Fig. 5). Further experiments to define relationship between T-antigen expression and Old-35 expression in IDH4 cells are in progress. Old-35 and p21 are coordinately expressed in

quiescent cells since many of the genes involved in terminal differentiation and senescence are predominantly active during the G_1 phase of the cell cycle, we determined whether Old-35 was expressed at this point of the cell cycle. To
5 achieve this objective, human diploid fibroblasts were grown to confluence (a classic way to arrest and synchronize these cells) (*Tseng et al., 1983). After release of the cells from confluence, following a short lag cells re-entered G_1 phase and then the cells traversed though S, G_2, M and back to G_1.
10 In these cells, Old-35 was highly expressed during the confluence period and at G_1 (Fig. 6). Additionally, as more of the cells entered G_1 Old-35 expression increased. After 15hr, Old-35 expression was significantly reduced, but expression increased again when the cells became confluent
15 (20 hr). The expression of p21 (G_1 specific cyclin-dependent kinase inhibitor) coincided with the expression of Old-35 (Fig. 6).

20 **STABILITY OF OLD-35 IN IFN-B TREATED CELLS**

The 3' UTR of particular lymphokines, cytokines and proto-oncogenes contain ARE elements that are implicated in regulating mRNA stability (Fig. 7). The presence of four such ARE elements in the 3' UTR of Old-35 suggests that mRNA
25 stability may contribute to differential expression of this gene under varied treatment and growth conditions. Recently, HuR a protein involved in the destabilization of mRNAs containing ARE elements has been purified and identified as a member of the Elav-line gene family (Myer et al., 1997).
30 If the HuR protein can regulate the stability of Old-35 in HO-1 cells, then treatment of cells with cycloheximide, which inhibits protein synthesis, should decrease or eliminate the HuR protein thereby resulting in stabilization of Old-35 mRNA. Cycloheximide treatment of HO-1 cells (Fig. 8A, lane
35 2) and IFN-b pre-treated HO-1 cells (Fig. 8A, lane 6 and 7) increases the level of Old-35 mRNA indicating that factors responsible for its degradation might have been inhibited. The cycloheximide studies also indicate that induction of

Old-35 expression can occur in the absence of new protein synthesis (Fig. 8A, lanes 3, 4 and 5). However, since Old-35 RNA production in HO-1 cells occurs within 3 hr of treatment, and cycloheximide is present for the entire treatment period,
5 it is possible that modifications of existing proteins may occur prior to changes in Old-35 transcription. Control of mRNA levels in a cell are regulated predominantly at two points: transcription and mRNA stability. To determine if IFN-β + MEZ or IFN-β effect Old-35 mRNA stability in HO-1
10 cells, the half-life of the Old-35 mRNA was determined as previously described (Jiang et al., 1993b) (Fig. 8B). Untreated and IFN-β + MEZ or IFN-β treated HO-1 cells were incubated with the RNA polymerase II inhibitor Actinomycin D (Act D) and the stability of pre-existing mRNA was
15 determined by Northern blotting. This experiment revealed that the half-life of Old-35 mRNA in HO-1 cells treated with IFN-β + MEZ or IFN-β is ~6-8 hr, suggesting that stabilization of this mRNA may contribute to the elevation of Old-35 levels in treated cells (Fig. 8B and data not
20 shown). However, because of the low level of Old-35 expression in untreated HO-1 cells, it was not possible to accurately determine the half-life of this message in these cells. Whether the observed low levels of Old-35 mRNA in untreated actively proliferating HO-1 cells are the result
25 of a lack of transcriptional activation or mRNA stability still remain to be determined. Nuclear run-on assays, that measure rate of RNA transcription, should reveal whether the promoter is active in HO-1 cells in the absence of IFN-β treatment and the potential contribution of transcriptional
30 activation to elevated Old-35 mRNA following IFN-β and IFN-β + MEZ treatment.

CLONING AND SEQUENCE ANALYSIS OF OLD-35

An initial 600bp fragment of Old-35 was identified and cloned
35 from a differentiation inducer treated subtracted (DISH) HO-1 cDNA library as described in the library screening protocol. This cDNA was cloned in a pBlueScript vector in the opposite orientation 3'-5' (EcoRI-XhoI) as a result of subtraction

hybridization. During the subtraction procedure, cDNAs are excised from the vector by double-digestion with EcoRI and XhoI. Since many cDNA also contain internal EcoRI-XhoI sites, many cDNAs will be cut internally and after the subtraction
5 procedure they will re-ligate in the incorrect direction. Thus the original 600bp fragment of Old-35 contained an internal region of Old-35 cDNA and lacked 3' and 5' flanking sequences. The 5' region of Old-35 was cloned from IFN-β treated HO-1 cells using a recently developed cDNA extension
10 procedure, complete open reading frame cloning (C-ORF), yielding in a single-reaction an ~2kb fragment (Kang and Fisher, unpublished). The 3' region of Old-35 was cloned using the 3' RACE procedure with 3' gene specific nested primers and dT, yielding an ~400bp product. The final
15 sequence of Old-35 is shown in Fig. 9. Although a portion of the 5' may still be missing, the Old-35 cDNA obtained using C-ORF and 3' RACE represents a near full-length clone judging from the Northern blotting data (Fig. 1), in which Old-35 hybridizes with an ~2.4-2.7 kb RNA species. Sequence
20 analysis revealed that the Old-35 cDNA (~2.6kb) contains a less frequently observed polyadenylation site (AUUAAA) (found in only ~10% of cDNAs) (Manley et al., 1988). The putative protein sequence does not exhibit homology to any known genes except to the *Escherichia coli* PNPase (polyribonucleotide
25 phosphorylase) gene of which 30% of the sequence is homologous and 50% displays sequence similarity (Fig. 10).

EXPERIMENTAL DISCUSSION

Controlled cellular proliferation is paramount for sustaining
30 homeostasis in multicellular organisms. The regulation of this dynamic process is of particular relevance in maintaining a balance between cell loss and cell renewal, important factors in development, differentiation and aging. Moreover, abnormalities in cell division are hallmarks of
35 many disease states, including developmental and congenital birth defects, premature aging syndromes and abnormal proliferative states such as cancer. Several genes involved

in cell proliferation control, including the tumor suppressor p53 and the cyclin dependent kinase (cdk) inhibitor p21, display elevated expression in growth suppressive conditions, such as quiescence (Niculescu et al., 1998, Lacombe et al.,
5 1996, Linke et al., 1996), senescence (Irving et al., 1992; Gire and Wynford-Thomas, 1998) and terminal cell differentiation (Jiang et al., 1994b, 1995b; Steinman et al., 1994). Since both terminal differentiation and senescence are characterized by growth arrest, it is possible that similar
10 and overlapping genes and gene expression changes may mediate these processes. To test this hypothesis we have screened a subtracted differentiation inducer treated human HO-1 melanoma library with mRNA derived from senescent human fibroblasts. This approach has resulted in the isolation of
15 a large number of cDNAs, consisting of both known and novel sequences (Table 1), displaying elevated expression in senescent human fibroblasts. Several of the same cDNAs, have also been independently identified from the same subtracted HO-1 library after screening with mRNA isolated from HO-1
20 cells treated with IFN-b + MEZ that induce irreversible growth arrest and terminal differentiation (Huang et al., 1999). This observation validates our hypothesis and suggests that this novel approach may prove useful in identifying and cloning genes displaying coordinated expression as a function
25 of induction of growth arrest during terminal differentiation and cellular senescence. One such cDNA is the novel gene, Old-35.

Induction of terminal differentiation in human melanoma cells
30 by IFN-β + MEZ frequently results in the induction and up-regulation of genes that also display elevated expression following exposure to IFN-β, referred to as Type I melanoma differentiation associated (mda) genes (Jiang and Fisher, 1993). Old-35 represents such a gene, since its expression
35 is elevated in HO-1 cells after treatment with IFN-β and IFN-β + MEZ. Old-35 is also up-regulated during growth arrest and senescence in human fibroblasts, indicating that its expression is not restricted to only programs of

differentiation or to human melanoma cells. Since IFN-β has well-established antiproliferative properties, in both normal and cancer cells (Fisher and Grant, 1985), it is possible that Old-35 may function as a down-stream gene in the

5 IFN-signaling pathway culminating in growth arrest. A number of experiments indicate that Old-35 expression is related to cellular senescence and proliferative quiescence. Analysis of Northern blots from young versus senescent human fibroblasts indicates restricted expression of Old-35 to

10 senescent cells. IDH4 cells, conditionally immortalized by a DEX-inducible SV40 T-antigen, represent an excellent *in vitro* model to study senescence (Wright et al., 1989). The presence of DEX in the growth media allows the IDH4 cells to actively proliferate, while the absence of it causes them to

15 senesce. In these cells, Old-35 expression is only detected after 7 days of growth in media devoid of DEX. This expression also corresponds with the SA- (-GAL staining of IDH4 cells, a well-established senescence marker (Dimri et al., 1995). Old-35 expression also increases when fibroblasts

20 become arrested in G_0 by growth and maintenance at confluence. In these contexts, Old-35 could prove useful as a diagnostic marker for cellular senescence, terminal differentiation and growth arrest. High levels of Old-35 expression are also found in the brain and heart, the only

25 human tissues that do not possess active regenerative properties. Judging from the localized expression of Old-35 during development, this gene may contribute to heart and brain development by assisting in the maintenance of terminal differentiation of cells in these organs. Due to the high

30 sequence homology of Old-35 to bacterial polyribonucleotide phosphorylase (PNPase), it is possible that Old-35 protein may exhibit a PNPase enzymatic activity. PNPase is one of the critical components of the *Escherichia coli* RNA degradosome (Blum et al., 1997), which consists of both PNPase and

35 endoribonuclease RNase E. The function of this complex is to control the rate of mRNA degradation. The PNPase possesses two enzymatic activities, 3'-5'processive exoribonuclease

activity and 5'-3' RNA polymerase activity (Blum et al., 1997). Recently, it has been shown that PNPase also has the capacity to bind to a specific double-stranded DNA sequence in a sequence-specific manner (Zhang et al., 1998). Since

5 Old-35 is differentially expressed in cells that undergo growth arrest, it is possible that this gene may play a role in RNA degradation in growth arrested cells. Additionally, since genes containing AUUUA elements (Myer et al., 1997) have been shown to be involved in the global regulation of

10 gene expression it is possible that Old-35 by binding sequence-specific targets, controls growth related gene expression. In this context, Old-35 might display tumor suppressor properties and could be useful for the gene therapy of cancer.

TABLE 1

CLONE DESIGNATION	CLONE IDENTITY
Old-1	Vimentin
Old-2	Human ribosommal protein S3a, v-fos
Old-5	mRNA M phase phosphoprotein
Old-7	RIG-G, Cig49
Old-11	MHC class I lymphocyte antigen
Old-14	Human non-muscle myosin alkaline light chain
Old-18	Human ADP-ribosylation factor 4
Old-19	Human mitochondrial cytochrome oxidation
Old-24	56 kDa IFN inducible
Old-30	Ribosommal protein L5
Old-32*	Novel*
Old-34	IFN-inducible protein
Old-35*	Novel*
Old-38	H.s. small acidic protein
Old-39	Human acidic ribosomal phosphatase
Old-42	Neurofibromatosis type 1

Old-59	Human nuclear receptor hTAK1
Old-60	Mitochondrial DNA
Old-61	Transcription factor I (99%)
Old-64*	Novel*
Old-65	CDC16HS cell 81, 261-68
Old-74	Human ISG 54K gene (IFN-gamma)-cig42
Old-79	Human T-complex polypeptide I gene
Old-80	Vitamin D induced
Old-83*	Novel*
Old-87*	Novel*, Possibly similar to Old-83
Old-107*	Novel*-Human homologue of Cow G-Protein
Old-113	DNA binding protein
Old-115	U1 small nuclear RNP
Old-119	Human HS1 protein
Old-121*	Novel*
Old-137*	Novel*
Old-139*	Novel*

Old-140	Human putative trans. CA150
Old-142*	Novel*
Old-144	MLN70 calcium- binding
Old-165	T-cell cyclophilin
Old-170	Human homologue of rat zinc transporter
Old-175 (5-3)*	Novel*

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Example #1

Background and significance

During terminal differentiation and senescence many genes are differentially expressed. Two processes that
5   control the overall mRNA levels are transcription and mRNA stability. Since both proliferation and differentiation are dynamic processes requiring continuos regulation (Blau, H.M., 1992, Blau et al., 1992, Blau et al, 1985) a thorough knowledge of the
10   molecular mechanisms that regulate gene expression will significantly contribute to our understanding of development, differentiation and malignancy. Gene expression is regulated by two mechanisms: transcriptional mechanisms which determine the rate of
15   mRNA production and equally important but under-studied post-transcriptional mechanisms which determine the overall amount of protein being produced. The experimental data from *Xenopous laevis, Drosophila melanogaster, Caenorhabditis elegans* document the
20   importance of post-transcriptional mechanisms in early patterning of the embryos which directs correct distribution, stability, and translation of inherited maternal transcripts (Seydoux, G., 1996) Additionally, in plants, it has been shown that it is the post-
25   transcriptional regulation and not transcription that directs the differentiation of chloroplast from its protoplast precursor (Deng and Gruissem, 1987). In mammals, posttranscriptional regulation appears to be important in cells responding to environmental stress,
30   proliferation and differentiation (June et al, 1990,Sierra et. al., 1994)

The sequences responsible for post-transcriptional regulation are found in the 3' untranslated regions (3'UTR) of the transcripts. When orthologous genes were compared,
35 large regions were found to exhibit more than 70% conservation over 300-500 million years of evolution, from mammals, birds, amphibians, or fish (Spicher et al., 1998).

Post-transcriptional regulation of mRNA levels is a pivotal control point in gene expression. Early response genes, such as cytokines, lymphokines and proto-oncogenes are regulated by a cis-acting adenylate-uridylate-rich element

5 (ARE) found in the 3' untranslated region (UTR) of the mRNA (Caput *et al.*, 1986; Shaw and Kamen, 1988; Chen and Shyu, 1995; Myer *et al.*, 1997). Currently, three classes of destabilizing elements have been identified: AUUUA-lacking elements and AUUUA-containing elements grouped into those

10 with scattered AUUUA motifs (such as proto-oncogenes) and those with overlapping AUUUA motifs (such as growth factors) (Chen *et al.*, 1995; Myer *et al.*, 1997). A replacement of 3'UTR containing ARE in place of a 3'UTR of a stable message, such as β-globin or luciferase targets

15 this very stable mRNA for rapid degradation (Shaw and Kamen, 1988, Maddireddi et al., 2000). In contrast, the removal of an ARE stabilizes an otherwise labile message (Miller *et al.*, 1984; Lee *et al.*, 1988)

A pool of genes involved in mRNA stability remains very

20 small. However, one of the best studied family of genes in this area is Elav. Elav, which stands for embryonic -lethal abnormal vision, was first identified in *Drosophila melanogaster*. Deletion mutants of the *elav* gene are embryonic lethal because of lack of neuronal

25 differentiation (Robinow and White, 1991). In mammals and in Xenopus, the elav gene family consists of three members that are developmentally regulated and tissue specific (Hel-N1, HuC, HuD,) and one member that is ubiquitously expressed called HuR (Szabo et al, 1991, Good, 1995, Ma et

30 al., 1996,Antic and Keene, 1997). The mechanism by which Elav genes promote the differentiation of neurons is not completely understood, however, it is known that Elav can bind AU rich elements in ther 3'UTRs of selected genes. By selectively stabilizing selected genes, the overall amount

35 of gene expression changes observed during terminal differentiation of neurons is regulated.

To obtain further insights into 3' UTR stabilization, 3'
end maturation has been studied in detail in plants and
bacteria. It is worth noting that the protein complexes
involved in this process in these two completely different
5 organisms are highly conserved. They are composed of
endonucleases, exonucleases, helicases and enolases.
E.coli, which lives in an energy high environment, has two
exonucleases involved in the processing of 3'UTRs: RNase
II, which has hydrolytic activity and PNPase
10 (polynucleotide phosphorylase) which has phosphorolytic
activity (Higgins et al., 1993). Single mutant of either
PNPase or RNase II is viable, whereas double mutants die
(Donovan and Kushner, 1986). On the other hand, *B.Subtilis*,
which lives in the soil-an energy poor environment-
15 exclusively uses PNPase and lacks RNase II. There may be a
few different explanations for the presence of two
exonucleases in *E.coli*. Firstly, the two exonucleases may
have different specificities. This is supported by the fact
that a specific degradation of S20 mRNA accumulates in pnp
20 mutants but it fails to accumulate in rnb (Rnase II)
mutants (Mackie, 1989). Another explanation could be that
PNPase is phosphorolytic and Rnase II is hydrolytic. As
phosphorolysis releases nucleotide diphosphates (Gedefroy-
Colburn and Grunberg-Manago, 1966), the energy of the
25 phosphate bond is conserved. Differential use of these two
enzymes may reflect an adaptation to changing energy
conditions (Deutscher and Reuven, 1991). This model is
supported by the fact that *B. subtilis,* which normally
inhabits low energy environment, uses PNPase exclusively,
30 while *E.coli* predominantly uses Rnase II. Another
interesting point worth noting is that PNPase also
functions during competence development of *B.subtilis* .
Since compentence is a state during which specialization is
acquired, competence has been used as a simple model for
35 differentiation. Genetic competence may be defined as a
physiological state enabling a bacterial culture to bind
and take up high-molecular-weight exogenous DNA

(transformation). The study of competence genes has permitted their classification into two broad categories. Late competence genes are expressed under competence control and specify products required for the binding, 5 uptake, and processing of transforming DNA. Regulatory genes specify products that are needed for the expression of the late genes (Dubnau,1991). PNPase is necessary for the expression of late competence genes. Transformability of pnp mutant is 1-5% of that seen in wild type strains 10 (Luttinger A, et al.,1996)

In plants, PNPase functions during chlororoplast differentiation where it is involved in processing of plastid 3' UTR (example:petD). It is interesting to note 15 that plastid genes also possess AU rich regions in its 3' UTR. Identically to bacteria, plant PNPase has a 3'-5' processive exonuclease activity that exhibits increased specificity for poly(A) and poly(U). (Hayes et al., 1996) Human teratocarcinoma cells (NT2) can be differentiated 20 into neurons with retinoic acid treatment and thus provides an excellent model to study neuronal differentiation. Recently it has been shown that a member of elav family, Hel-N1, when transfected into NT2 cells, forms neurites, an early sign of differentiation. However 25 it does not cause terminal differentiation (Antic et al., 1999). Since Old-35 encodes PNPase, a 3'-5' exonuclease involved in degradation of mRNA sequences, it is possible that Old-35 can increase the effects of Hel-N1 in NT2 cells and cause them to differentiate.

30

Determining the half-life of Old-35 mRNA in HO-1 cells

Since Old-35 has an AU rich 3'UTR (Fig.4) we have speculated that its expression may be regulated by post-transcriptional mechanisms. One way to study post-35 transcriptional processes is to investigate mRNA half-lives. In a mammalian cell culture system this can be achieved by treating cells with Actinomycin D (AD). Since

AD inhibits RNA polymerase II activity, mRNA synthesis is terminated and the mRNA synthesized before AD treatment is allowed to decay. Total RNA is collected at different time points and quantified using Northern analyses. Using this
5   protocol, we have examined the half-lives of Old-35 mRNA in HO-1, confluent HO-1, IFN-βtreated, and IFN-β+MEZ treated HO-1. The half-life of Old-35 in all the treatments did not change and **(Figure 11)** was estmated to be 6 hr. Since there was no difference in half-life between HO-1 and IFN-β
10 treated HO-1 it is assumed that a post-transcriptional mechanism is not responsible for the upregulation of Old-35 mRNA level in IFN-β treated HO-1.

Expression of Old-35 during growth arrest and senescence of
15 **IDH4 and AR5 cells**

IDH4 cells were produced by transfecting IMR-90, normal human fibroblasts, with a dexamethasone (DEX) inducible mouse mammary tumor virus-driven simian virus 40 T-antigen (Wright *et al.*, 1989). In this model system,
20 prolonged proliferation and the absence of markers of senescence are dependent upon the continued presence of DEX and thus the SV40 T-antigen. (Wright *et al.*, 1989). In DEX-free medium, DNA synthesis declines by ~80% within the first 3 days and reaches a minimum level at day 7. This
25 decline corresponds with a decrease in telomerase activity and T-antigen expression (Holt *et al.*, 1996). Since T-antigen has a long half-life (~3 days) and remains in the cells for about 5-7 days after the removal of DEX, it is possible that the up-regulation of Old-35 by day 7
30 corresponds with the depletion of T-antigen in these cells (**Figure 12**). However, there is one drawback associated with using IDH4 cells. Since the expression of T-antigen is dependent upon DEX, a shift of IDH4 cells towards senescence is dependent upon a complete depletion of DEX
35 from the media and serum in which the cells are growing. This is normally achieved by charcoal stripping of the

serum. However, since fetal serum contains vast amount of steroids, it becomes a challenge to do so in completion. Thus, the reproducibility of complete DEX depletion is a problem. To overcome this problem, we used another cell line, AR5. AR5 is very similar to IDH4, except the fact that T-antigen is not DEX inducible but it is rather temperature sensitive. AR5 cells are able to grow rapidly at 35°C since they are expressing T-antigen. When shifted to 39°C, T-antigen is degraded and the cells become senescent. Total RNA was collected from AR5 cells grown at 35°C and from AR5 cells shifted to 39°C. Old-35 was expressed one day after the shift and at the later time points as well **(Figure 12)**. To make sure that the cells had reached senescence when shifted to 35°C, we hybridized the Northern blot to the well characterized senescence marker, p21 (CDK inhibitor) **(Figure 12)**. p21 expression increased in AR5 cells shifted to 39°C and showed a pattern similar to Old-35.

The difference between expression of Old-35 in IDH4 and AR5 cells can be accounted for by the differences in T-antigen depletion. Since T-antigen degrades much faster in AR5 cells (temperature sensitive) than in IDH4 cells (half-life 2-3 days), AR5 cells reach a senescent state at much faster rate than DEX depleted IDH4 cells.

Cloning of the second variant (3.8 kb)

Once most of the sequence was known, the cDNA was screened against the BLAST·EST database. In this search we have identified another version of Old-35 (3.8 kb) which is probably the upper band observed on Northern blots. The 3.8 kb EST was sequenced. The sequence analysis revealed differences in 3' UTRs of the 2.6kb and 3.8kb fragments. This may result from different polyadenylation patterns. To make sure that the upper band on the Old-35 Northern Blot represents the 3.8kb fragment, we will use the 3' UTR of the ATCC clone as a probe (Figure 13)

Old-35-GFP localization

Since no antibody for Old-35 is currently available, we decided to test the localization of Old-35 by creating an N-terminal fusion of Old-35 and GFP (Clontech). Old-35
5 was cloned in frame with GFP without the first ATG and then transfected into HeLa and HO-1 cells with SuperFect reagent (Clontech). The protein was allowed to express for 24hr. As expected for a degradative enzyme, Old-35 localized to the cytoplasm of Hela (Figure 14) and HO-1(data not shown).
10

Expression of Old-35 during mouse development

Using Human Multiple Tissue Northern (MTN) Blots (Clontech) we determined that Old-35 was expressed in all of the tissues tested with the highest levels in the heart
15 and brain. Since terminal differentiation of specific tissue cell types occurs during normal development of the embryo, the expression pattern of Old-35 was determined during mouse development. The highest level of Old-35 expression was apparent during the earliest stage of
20 development (8 days) and it steadily declined with time (10 to 16 days). To determine spacial expression of Old-35, in situ hybridization expreriments were performed. Murine Old-35 was expressed in the spinal tube and in the arteries. However more expreriments have to performed to correctly
25 determine the expression pattern (Figure 15)

Effect of different interferon-α subtypes on Old-35 expression of Old-35

All subtypes of IFN-α stimulated Old-35 expression.
30 IFN-α H and αI-stimulated Old-35 in the lowest extent. (Figure 16). The above experiments document differential regulation of Old-35 expression by different cytokines, with type I interferons (IFN--α /IFN-β) being the most active cytokines tested in inducing Old-35 expression in
35 HO-1 cells. Moreover, since IFN signaling cascades

include Jak and Stat activation, they may prove to be important intermediates of Old-35 induction and expression.

5 Old-35 genomic structure

As described above, we have identified two BACs that were 100% homologous to specific regions of Old-35 cDNA. First BAC (RPCI-11, Plate=702,Col=8, Row=C) (Research Genetics) showed 100% homology to the 2207-
10 2365 region of Old-35 cDNA. The sequenced regions flanking the Old-35 sequence were foreign thus it is highly possible that they are introns. The second BAC (CITBI-E1, clone 2505G20) (Research Genetics) showed 100% homology in 235bp-313bp region of Old-35 cDNA.
15 After sequencing of the BACs, it became apparent that the Old-35 gene is distributed among 28 exons (Table 2).The spaces in the intron column signify no data for the intron size. The intron sizes are being determined.

Interestingly, there are at least three pseudogenes
20 of Old-35 in the human genome. The first one is 92% homologous to the Old-35 cDNA and contains a portion of the cDNA (48bp-1387bp). 5' and 3' ends of the cDNA could not be found on this BAC. The second pseudogene is present on the 3rd chromosome as determined by BLAST
25 search at it contains a cDNA fragment from the 49th nucleotide to the end of cDNA. This pseudogene exhibits 92% homology to the Old-35 cDNA. The third pseudogene also contains a cDNA fragment from 49 bp to 2517 bp. The second and third BACs are 90% homologous. In all cases,
30 all of the BACs are highly mutated and intronless parts of the Old-35 cDNA.

35

TABLE 2

EXON-INTRON STRUCTURE OF OLD-35

exons	exon size	introns	intron size
1	174	1	6000
2	60	2	1100
3	74	3	1300
4	105	4	1100
5	49	5	
6	63	6	
7	47	7	
8	113	8	6600
9	186	9	800
10	51	10	600
11	57	11	3500
12	96	12	800
13	102	13	
14	70	14	

exons	exon size	introns	intron size
15	36	15	
16	66	16	
17	89	17	
18	53	18	
19	105	19	
20	72	20	
21	63	21	
22	83	22	
23	83	23	
24	106	24	
25	55	25	
26	77	26	
27	45	27	
28	406		